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14. ABSTRACT: HER2 overexpression is a poor prognostic indicator in breast cancer. HER2 amplification is associated with early tumor dissemination, rapid tumor progression, and increased invasiveness, implying that HER2 has a significant role in the metastatic phenotype. We have demonstrated that two key steps in the metastatic process, angioinvasion and transendothelial migration, are augmented by HER2 expression, and we have linked Angiopoietin-2, a vascular destabilizing protein, to expression of HER2 (1). The objective of this research is to determine if the metastatic advantage of HER2 expressing cancer cells is imparted by Angiopoietin-2 production, and further to determine if overexpression of HER2 up-regulates Angiopoietin-2 expression. The scope of this research was to test: 1) angioinvasion using an in vitro microvessel dismantling assay and 2) endothelial cell retraction, a key step in tumor-cell transendothelial migration. We tested HER2 amplified breast cancer cell production of Angiopoietin-2, using blockade or stimulation of HER2 signaling. To determine if Angiopoietin-2 modulates the metastatic steps in question, we applied Angiopoietin-2 directly, or sequestered Angiopoietin-2 in these models. Breast cancer specimens were also tested for correlation of expression of HER2 and Angiopoietin-2. Further, we identified mechanistic steps in HER2 regulation of Angiopoietin-2 in breast cancer cells. Lastly, we demonstrated that the mechanism of HER2 amplified breast cancer cell-induction of endothelial cell retraction involves downregulation of VE cadherin and dissociation of catenin proteins from VE cadherin. This process releases the adherins junctions of the endothelial monolayer, thereby disrupting endothelial integrity.

15. SUBJECT TERMS
Angiopoietin 2, HER2, Regulation of function, breast cancer metastasis, mechanism of angio invasion

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Introduction

HER2 overexpression is a poor prognostic indicator in breast cancer. HER2 amplification is associated with early tumor dissemination, rapid tumor progression, and increased invasiveness, implying that HER2 has a significant role in the metastatic phenotype. We have demonstrated that two key steps in the metastatic process, angioinvasion and transendothelial migration, are augmented by HER2 expression, and we have linked Angiopoietin-2, a vascular destabilizing protein, to expression of HER2 (1). The **objective** of this research is to determine if the metastatic advantage of HER2 expressing cancer cells is imparted by Angiopoietin-2 production, and further to determine if overexpression of HER2 up-regulates Angiopoietin-2 expression. The **scope** of this research was to test: 1) angioinvasion using an in vitro microvessel dismantling assay and 2) endothelial cell retraction, a key step in tumor-cell transendothelial migration. We tested HER2 amplified breast cancer cell production of Angiopoietin-2, using blockade or stimulation of HER2 signaling. To determine if Angiopoietin-2 modulates the metastatic steps in question, we applied Angiopoietin-2 directly, or sequestered Angiopoietin-2 in these models. Breast cancer specimens were also tested for correlation of expression of HER2 and Angiopoietin-2. Further, we identified mechanistic steps in HER2 regulation of Angiopoietin-2 in breast cancer cells. Lastly, we demonstrated that the mechanism of HER2 amplified breast cancer cell-induction of endothelial cell retraction involves downregulation of VE cadherin and dissociation of catenin proteins from VE cadherin. This process releases the adherins junctions of the endothelial monolayer, thereby disrupting endothelial integrity.

Body

Specific aim #1 was to determine if production of Angiopoietin-2 by HER2 expressing breast cancer cells influences key metastatic steps of endothelial cell retraction, a key step in tumor cell transendothelial migration, and angioinvasion.

Task #1.

A. **HER2 signaling and Angiopoietin-2 influence on angioinvasion**. We tested Her2 signaling and Angiopoietin-2 in the mechanism of angioinvasion using a 3-dimensional in vitro model of intact rat microvessels embedded in collagen I gel. Angioinvasion was implied by breakdown of the microvessel integrity.

We showed that microvessels dismantle upon exposure to MCF-7 cells or HER2 overexpressing MCF-7 cells (HER). After coculture with these cells, matrix-embedded microvessels demonstrate areas of discontinuity, with architectural dismantling. We compared MCF-7 cells with HER cells, which express significantly more Angiopoietin-2. HER cells induced a significantly more rapid and more extensive effect on microvessel dismantling ($p < 0.05$ vs MCF-7). We then pretreated MCF-7 cells with trastuzumab or heregulin β1 to block or induce HER2 signaling. Manipulating HER2 signaling dose-dependently modified the tumor cell induced microvessel dismantling ($p < 0.01$). These results were also seen with other HER2 expressing breast cancer cell lines. We then tested the direct application of Angiopoietin-2 protein to induce microvessel dismantling,

and used sTie2/Fc to sequester tumor produced Angiopoietin-2. Microvessels were exposed to Angiopoietin-2 protein in increasing doses up to 200 ng/ml, without significant induction of microvessel dismantling. Pretreatment with sTie2/Fc dose-dependently-inhibited microvessel dismantling, reaching significance at 200 ng/ml ($p < 0.01$) although this effect was not dramatic. These results were presented as an oral abstract at the Society of University Surgeons Annual Meeting in February 2001 in Chicago, Illinois and published in Surgery (2).

B. **Tumor cell induced endothelial cell retraction.** We used an endothelial cell monolayer model to evaluate the effect of HER2 signaling and Angiopoietin-2 production in breast cancer cells on endothelial integrity. Loss of endothelial integrity is a key step in tumor cell transendothelial migration. We tested MCF-7 breast cancer cell-induction of endothelial cell retraction during co-culture, using parental MCF-7 cells or transfected MCF-7 cells that overexpress HER2 (HER cells). The endothelial cell model used intact, 5-day-old monolayers of human iliac vein endothelial (HIVE) cells or human dermal microvessel endothelial cells (HDMEC) in tissue culture. Heregulin β1 was used to stimulate Her2 signaling or trastuzumab to block Her2 signaling, and we tested the effect on these treatments on endothelial cell retraction. Further experiments tested whether Aniopoietin-2 can induce EC retraction.

We showed that HER cells induce a greater degree of endothelial cell retraction, determined by the number of tumor cells associated with endothelial cell retraction events, and the percent of subendothelial matrix that is exposed by retracting endothelial cells. We also showed that HER2 signaling induces endothelial cell retraction. MCF-7 or HER cells pretreated with trastuzumab or heregulin β1 greatly increased or arrested the amount of endothelial retraction induced by coculture with MCF-7 cells. These findings were published in the International Journal of Cancer in 2001 (3).

HER cells produce more Angiopoietin-2 than MCF-7 parental cells. We initially tested Angiopoietin-2 directly in escalating doses to the intact endothelial monolayers (HDMEC). We also tested MCF-7 cells that were pretreated with soluble Tie2/Fc receptor fusion protein (sTie2/Fc) to bind and sequester tumor cell-released Angiopoietin-2, preventing binding to Tie-2 receptors on the endothelium. Angiopoietin-2 significantly induced significant endothelial cell retraction, with a dose dependent response between 0.5 and 50 ng/ml, ($p < 0.001$ vs MCF-7) [Figure 1]. Taken together, these data strongly suggest that Aniopoietin-2 production in MCF-7 cells, influenced by Her2 signaling, induces EC retraction. This conclusion would suggest that Aniopoietin-2 action might be a therapeutic target to arrest the metastatic phenotype in some breast cancers. In support of this hypothesis, recently published research demonstrates that Aniopoietin-2 sequestration eliminates angiogenic induction in corneal angiogenesis model, and promotes tumor regression in a xenograft model (4). These data were presented at the Era of Hope in 2002 and the American Association of Cancer Research in 2005, and a manuscript is in preparation for submission to the Journal of Surgical Research.

Because VEGF is known to increase vascular permeability, and is upregulated by Her2 signaling, we also tested VEGF in the EC retraction assay (5,6). VEGF alone did not induce EC retraction. The addition of VEGF to Aniopoietin-2 in the retraction assay significantly abrogated Aniopoietin-2 induced retraction ($p < 0.01$ Aniopoietin-2 50 ng/ml vs Aniopoietin-2 50 ng/ml + VEGF 50 ng/ml) [Figure 1]. This result is consistent with published data suggesting that Aniopoietin-2 alone promotes vascular destabilization, while the presence of VEGF with Aniopoietin-2 facilitates angiogenesis (7). This effect is evident at low-serum concentrations (2% FBS) but severely reduced at 15% FBS where there is no significant difference. We postulate that increasing levels of VEGF and Ang1 present in FBS modulate the effect in high serum. Increasing doses of sTie2/Fc to sequester Angiopoietin-2 significantly altered the ability of MCF-7 cells to induce endothelial cell retraction at a dose of 200 ng/ml sTie2/Fc ($p <0.05$ vs MCF-7) [Figure 1]. These data were presented at American Association of Cancer Research in 2005 and a manuscript is in preparation for submission to the Journal of Scientific Research.

C. Mechanism of HER2-induced Endothelial Cell Retraction These experiments were not described in the Statement of Work, but were designed to further elucidate the mechanism of HER2 signaling and Angiopoietin-2 induction of endothelial cell retraction. We postulated that endothelial cells retract after exposure to released factors from HER2 expressing breast cancer cells, specifically the binding of Angiopoietin-2 to the Tie2 receptor. We postulated that tumor cell supernatent or Aniopoietin-2 protein would induce the dissociation of the catenin proteins from vascular endothelial (VE) cadherin. These proteins are key structural elements of the adherens junctions of the endothelium, which maintain endothelial integrity. After exposure, a dissociation of α, β, and γ catenin from VE cadherin would break the adherens junction link to the cytoskeleton, resulting in retraction and rounding of the endothelial cell, and facilitate transendothelial migration of the tumor cells.

In these experiments, we tested intact human endothelial cell monolayers for dissociation of the catenins from VE cadherin after exposure to MCF-7, or MCF-7 cells pretreated with trastuzumab and heregulin β1 to manipulate HER2 signaling. We immunoprecipitated VE cadherin after exposure of the monolayer to tumor cells, and determined the quantity of the catenins that remained associated with VE cadherin by Western blot analysis. Western blots were digitized and the densitometric intensity was determined and compared to control. The data was reported as percent of control (untreated) monolayers. A time dependent loss of associated catenins was clearly demonstrated, with greater than 90% loss of catenin proteins seen at 24 hrs ($p < 0.01$). HER2 signaling regulation significantly altered the dissociation curve. A 50% reduction in γ catenin dissociation was seen at 24 hrs after treatment with trastuzumab. Heregulin β1 significantly augmented MCF-7 induced γ catenin dissociation ($p < 0.05$), achieving equivalence with the result of HER cell induction of catenin dissociation. We then tested the direct application of Aniopoietin-2 to EC monolayers to induce catenin dissociation. A dose- dependent dissociation of Aniopoietin-2 induced VE-cadherin from catenin proteins, maximized at 200 ng/ml of Aniopoietin-2, (beta: 64.7% of control, $p < 0.01$;

gamma: 78.2% of control, $p < 0.05$). Though significant, the effect of Aniopoietin-2 alone was not dramatic, and was not equivalent to tumor coculture.

We also sequestered Aniopoietin-2 with sTie2/FC from MCF-7 cells in coculture with endothelial monolayers. sTie2/Fc significantly reduced the γ catenin dissociation induced by MCF-7 cells back to 50% of control ($p < 0.05$ vs MCF-7), similar to trastuzumab treatment. These results imply that the mechanism of HER2 signaling induced endothelial cell retraction likely includes dissociation of the adherens junction proteins, with loss of continuity with the cytoskeleton. Further, these data implicate tumor cell produced Angiopoietin-2 as part of the mechanism of the HER2-signaling induced metastatic phenotype. These data were presented at the American Association of Cancer Research in 2005 and a manuscript is in final review, after revisions, to the Annals of Surgical Oncology (8).

Specific Aim #2 was to determine the concurrent expression of HER2 and Angiopoietin-2 in breast cancer specimens.

Pilot Study

In a pilot study of 11 cancers and 3 normal breast specimens, 5 cancers over-expressed HER2. Seven cancers expressed Aniopoietin-2, including all of the HER2 overexpressing cancers. Normal breast tissue did not express Aniopoietin-2. This pilot study was published in Surgery 2000 (1).

Using a tissue array, IHC analysis of 66 breast cancer specimens was performed for Aniopoietin-2 and HER2. Aniopoietin-2 staining was positive in 15 of 16 (93.7%) of HER2 positive cancers, and negative in 36 of 50 (72%) of HER2 negative cancers. The correlation coefficient (0.38182) was significant at $p<0.005$. This data was published in Cancer Research in 2007 (9).

Forty breast cancer specimens were collected for this study and subjected to Laser Capture Microdissection (LCM). Using RT-PCR, relative mRNA levels were determined for Aniopoietin-2 and HER2, and also for EGFR, HER3, HER4 and VEGF. Of these forty cancers, 17 were unevaluable, due to lack of sufficient cells within the specimen. In 23 specimens, LCM isolated breast cancer cells (eliminating vascular elements), RNA was isolated and RT-PCR performed in 13 ER+ cancers and 10 HER2+ cancer. Aniopoietin-2 mRNA was detected in all specimens. This result was not anticipated in the original proposal, but a recent publication demonstrated that Aniopoietin-2 appears to be linked to estrogen binding to the estrogen receptor in ER+ breast cancer cells lines (10). We performed a regression analysis of the data, which shows that the Aniopoietin-2 link to Her2 expression in Her2+ cancers is $R = 0.59$, $p < 0.07$. Although this correlation is not significant at the < 0.05 level, the study is underpowered to accept a negative conclusion. An additional study of 26 specimens would have sufficient power to detect a positive correlation at $p <0.05$. This proposed study would use improved LCM technology, and real-time PCR for quantization. This study was not able to be completed within budget. No correlation was seen with Her2 expression in the ER+ tumors. The

differences in the slopes of these two regression analyses are highly statistically significant. These data suggest that Aniopoietin-2 expression is linked to ER function in ER+ tumors (based on published data), but regulated by Her2 signaling in Her2 overexpressing cancers. These data are included in a manuscript in preparation for submission to the Journal of Scientific Research.

To determine the regulatory steps of HER2 signaling induction of Angiopoeitin-2, we tested breast cancer cell lines with trastuzumab and short interfering RNAs against HER2 to determine the signal pathways used in this mechanism. We demonstrated that inhibiting HER2 down-regulates Aniopoietin-2 production. We further demonstrated that the AKT and MAPK pathways are necessary for Aniopoietin-2 up-regulation by HER2. Stimulated Aniopoietin-2 production by heregulin B1 is abrogated by AKT and MAPK activity blockade.

These studies provide substantial evidence that the Aniopoietin-2 gene is regulated by HER2 activity in breast cancer, and proposes an additional mechanism for HER2 contributing to the tumor metastatic phenotype. These data were published in Cancer Research in 2007 (9).

Key Research Accomplishments

- Determined that HER2 signaling induces a metastatic phenotype in breast cancer involving endothelial cell retraction (as a key step in transendothelial migration) and microvessel dismantling (as a potential avenue of angioinvasion.)
- Determined that Angiopoietin-2 can induce endothelial cell retraction, and is likely a key factor in the mechanism of the HER2 signaling induced metastatic phenotype
- Determined that the mechanism of endothelial cell retraction involves dissociation of the catenin proteins from VE cadherin
- Determined that Angiopoietin-2 is likely involved in the mechanism of HER2 signaling induced microvessel dismantling, but is not the only factor in this mechanism.
- Identified that Angiopoietin-2 expression appears to correlate with Her2 expression in Her2 + breast cancers, although alternate-signaling pathways (ER) may also influence Angiopoietin-2 production in ER+ cancer cells.

- Identified that HER2 regulates Aniopoietin-2 production via AKT and MAPK pathways in breast cancer.

Reportable Outcomes

1. Carter WB, Ward MD. HER2 signaling-induced microvessel dismantling. Abstract. Presented at the Society of University Surgeons, February 10, 2001, Chicago, IL.
2. Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382-387.
3. Carter WB, Hoying JB, and Williams SK. HER2 overexpression enhances tumor cell transendothelial migration. Abstract. Presented at the Society of Surgical Oncology, March 12, 2001, Washington, D.C.
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5. Carter WB, Small G, Ward MD. Mechanism of Her2-induced endothelial cell retraction. Poster abstract. Presented at the Era of Hope, September 25-28, 2002, Orlando, FL.
6. Carter WB. Angiopoietin-2 induces endothelial cell retraction by dissociation of adherens junction proteins. Presentation at the AACR Annual Meeting, Anaheim, CA April 17, 2005 (Poster # 1154, Tumor Biology 10).
7. Carter WB. Her2 signaling in breast cancer induces dissociation of adherens junction proteins in endothelial cell monolayers. (Oral presentation). 1st Annual Academic Surgical Congress, San Diego, CA, February 2006.
8. Carter WB, Niu G. Human Epidermal Growth Factor Receptor 2 Regulates Angiopoietin-2 Expression in Breast Cancer via AKT and Mitogen-Activated Protein Kinase Pathways. *Cancer Research* 2007; 67 (4): 1487-1491.
9. Carter WB, Niu G, Ward M, Small G, Hahn J, Muffly B. Mechanisms of HER2-induced endothelial cell retraction. *Annals of Surgical Oncology*. Accepted August 2006, in final revision.

Conclusions

The work to date has substantially increased the knowledge available about the mechanisms involved in the development of a metastatic phenotype associated with HER2 overexpression. We have shown that at least two metastatic mechanistic pathways are enhanced by HER2 signaling; 1) endothelial cell retraction and transendothelial migration, and 2) microvessel dismantling as a portal for angioinvasion. Further, these metastatic pathways appear to involve Angiopoietin-2, a vascular destabilizing protein. The work presented identifies that the Angiopoietin-2/Tie-2 receptor pathway is likely a key intermediary step in the metastatic phenotype, and a worthy therapeutic target. Further, the determination of Angiopoietin-2 expression in breast cancer may suggest an appropriate tumor marker indicating greater metastatic or angiogenic potential.

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3. Carter, W. B., Hoying, J. B., Boswell, C., and Williams, S. K. (2001) HER2/neu over-expression induces endothelial cell retraction. *International Journal of Cancer* 91, 295-299

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Angiopoietin-2 induces endothelial cell retraction by dissociation of adherens junction proteins

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ABSTRACT

Tumor induced angiogenesis involving cooption of host vessels requires endothelial cell (EC) retraction and microvessel breakdown. Induction of Her2 signaling in Her2 expressing MCF-7 cells increases Angiopoietin-2 (Ang-2) production and enhances EC retraction. We postulated that tumor produced Ang-2 induces EC retraction by stimulating dissociation of adherens junction proteins (Vascular Endothelial (VE) cadherin and α, β, and γ catenins).

Using confluent human iliac vein endothelial (HIVE) cell monolayers, we treated EC with increasing doses of Ang-2 to induce EC retraction. Using immunoprecipitation, we tested EC for dissociation of catenins from VE cadherin after exposure to Ang-2 producing MCF-7 cells or Ang-2 protein in 2% FBS. Further, we manipulated Her2 signaling in these cells using Heregulin β1 or Trastuzumab to alter Ang-2 production and test for catenin dissociation. Lastly, we treated these cells with Tie2/Fc soluble receptor to quench released Ang-2 and tested for catenin dissociation.

Ang-2 producing MCF-7 cells induced a near complete dissociation of catenins from VE cadherin by 24 hrs. Dissociation was enhanced by Heregulin β1 ($p<0.05$) and decreased by Trastuzumab ($p<0.05$) treatments. Ang-2 (200 ng/ml) induced EC retraction ($p<0.001$ vs control), and dissociation of catenins from VE cadherin. This effect was completely abrogated by increasing FBS concentration to 15%. Sequestration of Ang-2 with sTie2/Fc (200 ng/ml) reduced EC retraction by 38% ($p<0.05$).

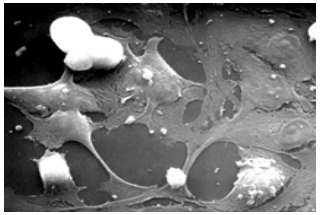
We conclude that Ang-2 induces EC retraction. The mechanism involves dissociation of the catenins from VE cadherin. Further, it appears that tumor cell produced Ang-2 may be a key factor involved in tumor induced angiogenesis and host vessel cooption.

INTRODUCTION

Angiogenic induction by tumor cells is multifactorial. VEGF appear to be essential to angiogenesis, inducing EC proliferation and migration. EC in mature, complex microvessel environments, however, are resistant to mitogenic stimulation by matrix adhesion, intercellular junctions, and pericyte stabilization. To disrupt the angiostatic state, ECs must be released from cellular and matrix adhesion. Release from cellular adhesion induces EC retraction by dissociation of alpha, beta and gamma catenins from VE cadherin at the adherens junctions of EC monolayers. Breast cancer cells can induce EC retraction by release of a soluble factor. This mechanism is enhanced in breast cancer cells overexpressing the HER2 protooncogene.

Angiopoeitin-2 is a key factor in angiogenesis, signaling in EC through the Tie2 receptor. Ang2 appears to function in the process of destabilization of microvessels by antagonizing the action of Ang1 by equal affinity binding to Tie2. In HER2 over-expressing breast cancer cells, HER2 signaling induces Ang2 upregulation and imparts an augmented ability to destabilize EC monolayers. We postulated that Ang2 production by tumor cells induces dissociation of adherens junction proteins as the mechanism of tumor-induced EC retraction.

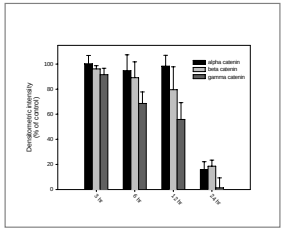
SEM of MCF-7 cells on HIVE monolayers showing EC retraction induced by Ang-2 producing cancer cells



3 hr 500X

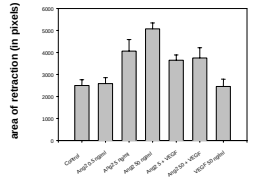
MCF-7 induced dissociation of Cadherin complex proteins

Anti-VE cadherin immunoppt of HIVE monolayers exposed to MCF-7 cells, and Western blot for catenins



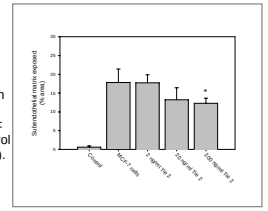
Endothelial Cell Retraction

HMEC monolayers treated with increasing doses of Ang2 for 24 hrs, with and without VEGF. The area of matrix exposed by retracting EC was quantified by image analysis (SigmaScan). Ang2 treatment induced EC retraction ($p < 0.01$ at 50 ng/ml vs control).



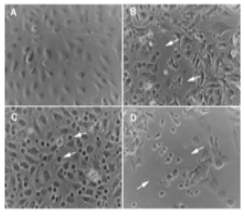
Soluble Tie2/Fc quenching of Ang-2

Blockade of MCF-7 induced EC retraction at 12 hrs by sequestration of Ang2 by sTie2/Fc ($p < 0.05$ vs control at 200 ng/ml).



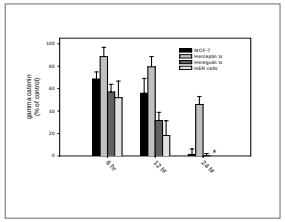
HER2 regulation of EC retraction

MCF-7 cells co-cultured with mature HIVE monolayers for 3 hr. Pretreatment with Herceptin to block Her2 signaling limited EC retraction, while treatment with Heregulin accelerated EC retraction.



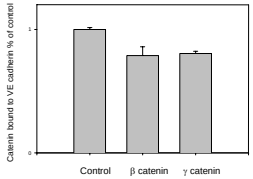
HER2 signaling control of Cadherin complex protein dissociation

Anti-VE cadherin immunoppt of HIVE monolayers after exposure to HER or MCF-7 cells pretreated with Heregulin β1 or Herceptin, with Western blot for catenins

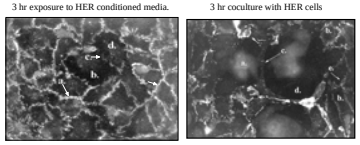


Ang2 induced Catenin dissociation from VE Cadherin

Ang2 induced dissociation of gamma and beta catenin from VE-cadherin in retracting EC after treatment with 200 ng/ml Ang2 for 24 hrs.



VE Cadherin stained HIVE monolayer

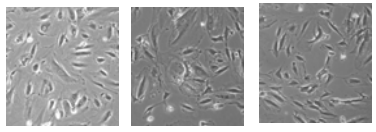


a. Normal VE cadherin staining
b. retraction gap
c. "free" EC membrane
d. EC

Mature EC monolayers showing redistribution of VE cadherin associated with EC retraction after coculture with MCF-7/HER cells or conditioned media.

a. MCF-7/HER cell
b. EC
c."free" membrane
d. EC retraction
e. Normal VE cadherin staining

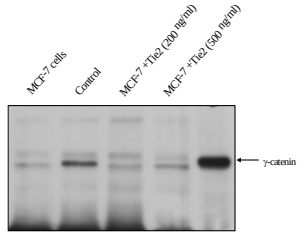
HIVE monolayers treated with Ang2 (200ng/ml)



2% FCS control

2% serum substitute control

Ang2 treatment in 2% serum substitute



γ catenin dissociation blockade by Tie2 soluble receptor sequestration of Ang-2.

RESULTS

1. A time dependent decrease in gamma and beta catenin immunoprecipitated with VE-cadherin was seen after coculture with MCF-7 or MCF-7/HER cells. ($p < 0.01$ at 24 hrs).

2. Manipulation of HER2 signaling with Herceptin or Heregulin β1 alter the ability of the cancer cells to induce EC retraction ($p < 0.05$ at 24 hrs.)

3. Ang2 protein induced EC retraction ($p < 0.05$ at 24 hrs.) The EC retraction induced by Ang2 was less (28% of control) than EC retraction induced by MCF-7 coculture.

4. Ang2 induced catenin dissociation from VE cadherin, consistent with the degree of EC retraction, but less dramatic than MCF-7 coculture (gamma catenin: 80.5% at 24 hrs, $p < 0.05$ vs control).

5. Sequestration of tumor cell produced Ang2 by treatment with sTie2/FC inhibited EC retraction by 38% ($p < 0.05$ vs control).

CONCLUSIONS

1. Ang2 production by breast cancer cells induces EC retraction.

2. The mechanism involves dissociation of catenins from VE cadherin, proteins that form the adherens junctions of EC monolayers.

3. Tumor cell produced Ang2 may be a key factor involved in tumor induction of angiogenesis.

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